NIST/NIH Vitamin D Metabolites Quality Assurance Program

Report of Participant Results

Winter 2010 Exercise

April 20, 2010

INTRODUCTION

The National Institute of Standards and Technology (NIST) has established a Vitamin D Metabolites Quality Assurance Program in collaboration with the National Institutes of Health (NIH) Office of Dietary Supplements (ODS). For our first exercise, participants were asked to determine the vitamin D metabolites, 25-hydroxyvitamin D, in three vials of human plasma (study sample). We requested that an individual concentration value for 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$) be provided along with a total concentration of 25-hydroxyvitamin D ($D_2 + D_3$) for each of three vials A, B, and C. Ethanolic calibration solutions with known concentrations of $25(OH)D_2$ and $25(OH)D_3$ were provided as control materials, and participants were also asked to provide measured values for the control solutions.

NIST has compiled and evaluated the data from the Winter 2010 exercise. Each participant is receiving an individualized report that summarizes their results relative to the other participants and to the expected values. Although a data summary is being distributed to all participants, laboratories were identified by code numbers known only to them.

While this report contains the formal results from the Winter 2010 exercise, we feel it would be useful to have an interactive meeting to discuss what was learned during this pilot exercise and to exchange information on methodology, calibration, etc. We have therefore arranged a Short Course titled "NIST/NIH Quality Assurance Program for 25-Hydroxyvitamin D Measurements," which will be held at the 2010 American Association of Clinical Chemistry (AACC) Annual Meeting and Clinical Lab Expo in Anaheim, California on July 28, 2010. We look forward to meeting the program participants and hope you will be able to join us in Anaheim.

We appreciate your participation in this pilot exercise. If you have any questions regarding this report, please contact us at vitdqap@nist.gov.

Sincerely,

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Research Chemists and VitDQAP Coordinators

OVERVIEW OF WINTER 2010 EXERCISE

In the Winter 2010 exercise, there were a total of 16 participants and 17 datasets (one participant provided data for two different methods). A summary of the individual participant data is provided in **Table 1.** The data include the average and standard deviation of the measurement of three vials (A, B and C) of human plasma (study sample) for $25(OH)D_2$, $25(OH)D_3$ and total 25-hydroxyvitamin D ($D_2 + D_3$) and a single measurement value for the ethanolic calibration solutions with known concentrations of $25(OH)D_2$ and $25(OH)D_3$ (238.6 ± 3.9 ng/mL and 334.8 ± 5.2 ng/mL, respectively).

Table 1. Summary of participant data for the Winter 2010 exercise.

	Human Plasma (Study Sample)[a]			*Ethanolic Controls*	
Participant Number	**$25(OH)D_2$ (ng/mL)**	**$25(OH)D_3$ (ng/mL)**	**total 25-hydroxyvitamin D ($D_2 + D_3$) (ng/mL)**	**$25(OH)D_2$ (ng/mL)**	**$25(OH)D_3$ (ng/mL)**
56	0.45 ± 0.07[b]	24.2 ± 0.3	24.5 ± 0.4	211	260
60	< 2	26.8 ± 0.3	26.8 ± 0.3	235	324
62			23.4 ± 0.9	176	340
160	< 1	27.6 ± 1.5	27.6 ± 1.5	237	329
180			26.0 ± 0.9		
182	< 2	26.3 ± 2.5	26.3 ± 2.5	262	368
183a	< 4	30.1 ± 1.0	30.1 ± 1.0	161	354
183b			29.6 ± 0.5	205	291
184		26.4 ± 2.3	26.4 ± 2.3	223	351
185		26.7 ± 0.3	26.7 ± 0.3	201	354
186		25.5 ± 2.0	25.5 ± 2.0	241	334
187	0.74 ± 0.07	24.8 ± 0.8	25.5 ± 0.7	214	347
188			27.0 ± 1.7		
189		29.2 ± 0.6	29.2 ± 0.6		
190			31.1 ± 1.2		
191			30.1 ± 0.2	310	320
192			23.7 ± 0.4		

[a]Average ± 1 standard deviation of n=3 replicate measurements
[b]Average ± 1 standard deviation of n=2 replicate measurements

Ten of these datasets originated from LC/MS/MS or LC/UV methods and 7 from immunoassay techniques (RIA, EIA or CLIA). **Table 2a** and **2b** summarize the LC and immunoassay methods, respectively, used by the participants.

Table 2a. Summary of LC methods used by VitDQAP Winter 2010 participants

Participant Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Detection*
56	$25(OH)D_2$-d_3 and $25(OH)D_3$-d_6	Samples were extracted with acetonitrile, centrifuged, then filtered	Online SPE with C8 column (2.1 x 30mm); separation on C18 column (2.1x50mm); step gradient methanol/water (86%/14% to 92%/8%); flow 0.5 mL/min	MS/MS MRM: $25(OH)D_3$ *m/z* 401/383; $25(OH)D_3$-d_6 *m/z* 407/389; $25(OH)D_2$ m/z 413/395; $25(OH)D_2$-d_3 m/z 416/398
60	$25(OH)D_3$ -d_6	IS solution was added to plasma (150 μL), and proteins were precipiated by addition of a ternary extraction solvent. Sample cetrifuged, supernatant transfered to new vial, dried, and dissolved in mobile phase	C-18 column (3.0 x 150 mm); A: 0.05 % formic acid in water, B: 0.05 % formic acid in methanol/acetonitrile (80/20, v/v); isocratic elution with 92% B from 0-2 min, step gradient to 100% B (2-8 min), equilibration (8-13) min; flow 0.55 ml/min	MS/MS (positive) MRM: $25(OH)D_3$ *m/z* 383/211; $25(OH)D_3$-d_6 *m/z* 389/211; $25(OH)D_2$ *m/z* 395/270
160	$25(OH)D_2$-d_6 and $25(OH)D_3$-d_6	Samples were extracted in acetonitrile, centrifuged and filtered	2-Dimensional separation CN column (2.1x 50mm) (1st Dimension), C18 column (2.1x 50mm) (2nd Dimension); gradient with 10mM formic acid in water and 10mM formic acid in methanol	MS/MS MRM: $25(OH)D_3$ m/z 401/365; $25(OH)D_3$-d_6 m/z 407/371; $25(OH)D_2$ m/z 413/337; $25(OH)D_2$-d_6 m/z 419/337
182	$25(OH)D_3$ -d_6	Proteins were precipitated with acetonitrile/methanol (3:1) and IS directly in 96 well plate. Plate was covered, mixed manually, and centrifuged	UPLC C18 column (2.1 x 50 mm); gradient elution from 60%-100% methanol with formic acid and ammonium acetate modifiers	MS/MS MRM: $25(OH)D_3$ *m/z* 401/365 (quant), *m/z* 401/383 (qual); $25(OH)D_2$ *m/z* 413/355 (quant), *m/z* 413/271 (qual)
183	$25(OH)D_3$ -d_6	IS (25 μL) was added to plasma (150 μL), followed by protein precipiation and extraction with 0.1 M $ZnSO_4$ (150 μL), methanol (300 μL), and hexane (750 μL); extract dried and dissolved with 70% methanol, 30% water with 2mM ammonium acetate	C8 column (2.1 X 50 mm); isocratic elution with 73% methanol/ 27% water; flow 0.4 mL/min	MS/MS MRM: $25(OH)D_3$ *m/z* 401/159, 401/383; $25(OH)D_2$ *m/z* 413/82, 413/395
184	$25(OH)D_3$ -d_6	Plasma (200 μL) extracted with acetonitrile and IS (700 μL); mixed, centrifuged, and filtered	C18 column (100 x 2.1mm; 5μm); linear gradient from 60% B to 98% B over 2 min (A: 0.1% formic acid in water, B: methanol with 0.1% formic acid and 5 mM ammonium acetate)	MS/MS (APCI) MRM: $25(OH)D_3$ *m/z* 383/257; $25(OH)D_3$-d_6 *m/z* 389/263; $25(OH)D_2$ *m/z* 395/209
185				MS/MS
186	$25(OH)D_3$ -d_6	Proteins were removed by addition of $ZnSO_4$, followed by methanol extraction and centrifugation; analytes were liquid/liquid extracted with three volumes of hexane, dried, and dissolved in methanol/water (65:35)	Phenyl UPLC column (2.1 x 100; 1.7um); 40°C; gradient: 65-85 % B over 3 min (A: 0.1% formic acid in water; B: 0.1% formic acid in methanol); flow 0.45 mL/min	MS/MS MRM: $25(OH)D_3$ *m/z* 401/159; $25(OH)D_2$ *m/z* 413/395
187				MS/MS
189	Obtained from kit supplier	Proteins were precipitated, samples centrifuged, analytes in the supernatant were extracted using SPE cartridges	Commercially obtained reagent set and column; isocratic elution; flow 0.7mL/min	UV at 265 nm

Table 2b. Summary of immunoassay methods used by VitDQAP Winter 2010 participants

Participant Number	*Immunoassay*	*Sample Preparation*	*Detection*
62	RIA	Samples were extracted, centrifuged	Gamma counter
180	RIA	Samples were prepared per vendor's sample extraction protocol	I^{125} detection using gamma counter
183	CLIA		
188	EIA		
190	EIA	Calibrators, controls and test specimens were all diluted with biotin-labeled 25(OH)D and analyzed in duplicate	Microplate reader
191	RIA		
192	EIA		Software was used to convert the absorbance values obtained from ELISA reader at 450 nm to get the concentration

Only the LC-based methods could distinguish between the two analytes of interest in the human plasma samples. All 10 of the LC-based datasets reported values for 25(OH)D_3, but only two reported values for 25(OH)D_2. The amount of 25(OH)D_2 in the human plasma was very low and below the detection limit for most of the LC-based methods. The 7 datasets from the immunoassay methods could not distinguish between 25(OH)D_2 and 25(OH)D_3 and reported the total 25-hydroxyvitamin D (D_2 + D_3) in the human plasma (study sample).

SUMMARY OF PARTICIPANT RESULTS

Your individual data as specified by your unique laboratory code is provided in **Table 3**, which contains information about your laboratory's performance relative to the other participants in this exercise and with respect to the NIST target value for the human plasma (study sample).

Table 3. Individual data table

Lab Code: NIST		Your Results			Community Results					NIST Analysis	
Analyte	Units	Mean	s_{total}	Z	N	Median	MADe	Min	Max	Value	U_{95}
25(OH)D_2	ng/mL	0.51			2	0.59		0.45	0.74	0.51	0.17
25(OH)D_3	ng/mL	24.3		-1.5	10	26.6	1.6	24.2	30.1	24.3	0.8
Total (D_2 + D_3)	ng/mL	24.8		-1.1	17	26.7	1.8	23.4	31.1	24.8	0.8

Mean: Average of your reported values
s_{total}: Overall standard deviation
Z: Z-score (Mean - Median)/MADe

N : Number of quantitative values reported
Median: Median of the reported values
MADe: Robust estimate of the standard deviation derived from the median absolute deviation (MAD)
Min, Max: Minimum and maximum reported values

Value: NIST-assessed value
U_{95}: ±95% confidence interval about the assessed value

Explanation of data in Table 3:

Your Results contains your individual laboratory results, including the mean and overall standard deviation (s_{total}) of the three data values provided for the human plasma (study sample). Also provided is a Z-score which is a measure of the difference between your individual result and the community's result; the significance of the Z-score is as follows:

- $|Z| < 2$ indicates that your result is within the consensus value of the community result
- $2 < |Z| < 3$ indicates that your result is marginally different from the consensus value of the community result
- $|Z| > 3$ indicates that your result is significantly different from the consensus value of the community result.

Please verify that the data in the table agree with your reported values.

Community Results contains the summarized results of all participants for the human plasma (study sample), which includes the total number of quantitative values reported (N), the median value for each analyte, the MADe (a robust estimate of the standard deviation), and the minimum (Min)/maximum (Max) values reported for the analyte.

NIST Analysis presents the NIST results for the human plasma (study sample). The expanded uncertainty, U_{95} (95% level of confidence), on the assigned value reflects within- and between-vial and between-method sources of variability. There is no evidence for inhomogeneity in this material for test portions of 450 µL or greater.

PROGRAM RESULTS AND DISCUSSION

For the ethanolic control solutions (SRM 2972), the single data values for 25(OH)D_2 and 25(OH)D_3 reported by each individual laboratory are plotted in **Figures 1a** and **1b**, respectively. The two primary methods of analysis (LC and immunoassay) are displayed separately with closed (●) and open (○) circles, respectively. For each of these graphs, the black solid line (——) represents the consensus median and the black dotted lines (- - - - -) represent the consensus variability (2 × MADe). The NIST-assessed value for this control material (SRM 2972) is provided by a red square (■) (with error bars representing ± U_{95}). The laboratories with results that fall between the two dotted lines are within the consensus variability area.

For each participant who used an LC method, the calculated average (mean) value and error bars (representing 2 × s_{total}) for 25(OH)D_3 in the human plasma (study sample) are plotted in **Figure 2.** The data for 25(OH)D_2 are not provided in graphical form because only two laboratories reported values that were above their detection limits. When the error bars for each data point are considered, all laboratory data are within the consensus variability (2 × MADe).

For all participant datasets, the calculated average (mean) values and error bars (representing 2 × s_{total}) for the total 25-hydroxyvitamin D ($D_2 + D_3$) in the human plasma (study sample) are plotted in **Figure 3.** From the mean values for all datasets, the consensus median and the consensus

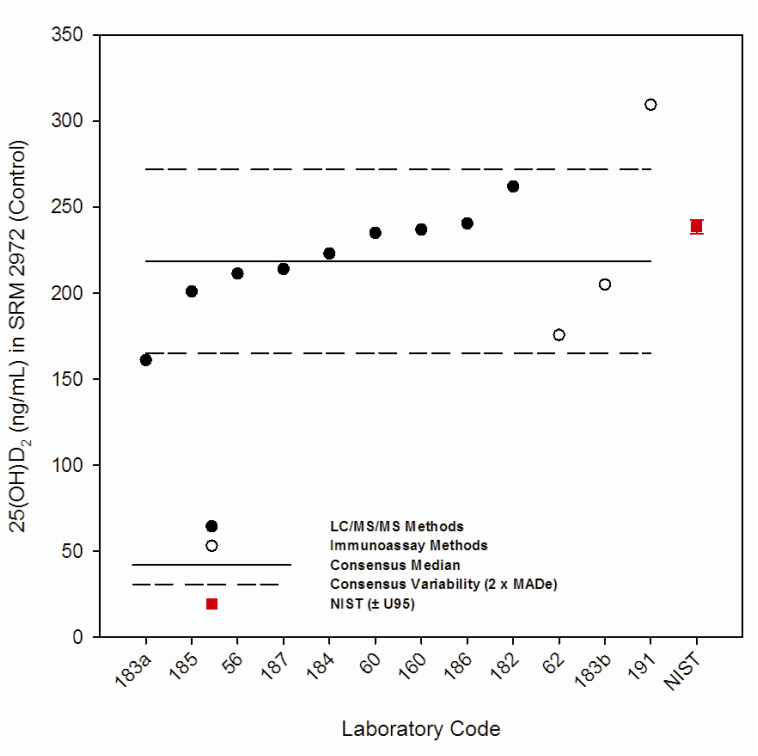
variability (2 × MADe) were determined. When the error bars for each data point are considered, all laboratory data are within the consensus variability. Both primary methods of analysis (LC, immunoassay) provide similar mean values and uncertainties for this human plasma (study sample).

The precision for the three replicate analyses of the human plasma (study sample) ranged from 2 % to 19 % for the individual laboratories, where over 2/3 of the laboratories that participated had method precision under 8 %. When all reported data for the human plasma (study sample) were considered, the consensus variability was ≈ 14 % and the consensus median was biased ≈ 7 % higher than the NIST-assessed value for this material. A goal for this program is to determine the cause of any bias and to achieve better agreement between the consensus median value and the NIST-assessed value. While the precision of most individual laboratories was good, another program goal is to reduce the consensus variability to better represent the community's measurement capability while also recognizing that a 'fit-for-purpose' variability-level may exist. One approach to meet this goal is to strive for individual laboratory precision of less than 10 %.

The complete exercise results for 25(OH)D_2 and 25(OH)D_3 are provided in **Figures 4a** and **4b**, respectively. The results for both the human plasma (study sample) and the ethanolic control (SRM 2972) for each participant are listed in tabular form and as a 2-D plot of ethanolic control data (x-axis) versus the human plasma (study sample) (y-axis). Individual laboratory results are displayed as lab-coded circles with corresponding errors bars (2 × s_{total}). Lab data points that are plotted on either edges of the graph (along either the x- or y-axis) have values for only one of the two test materials (control or study, respectively). The red box and the blue dotted-line box represent the range of NIST-assessed values and the consensus values, respectively, for the control and the study sample. These additional plots are provided to directly compare the individual laboratories' performance with respect to both plasma matrix samples and a non-matrix control material. For example, if your values are low (or high) for both the control and sample, you may have calibration issues. If your laboratory falls into this category, you may want to investigate how your calibrants are prepared, the purity of your calibrant material(s), and /or the method used to validate concentration (e.g., gravimetry vs. UV-spectrophotometry).

Figure 1. Winter 2010 exercise results for (a) 25(OH)D_2 and (b) 25(OH)D_3 in the ethanolic control (SRM 2972).

(a) 25(OH)D_2



(b) 25(OH)D_3

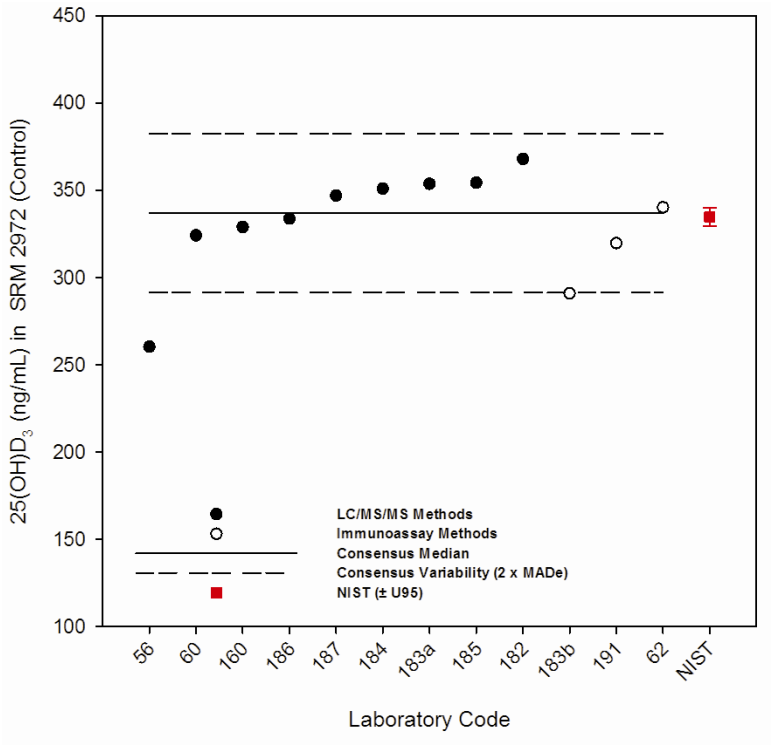


Figure 2. Winter 2010 exercise results for 25(OH)D_3 using only LC methods in the human plasma (study sample).

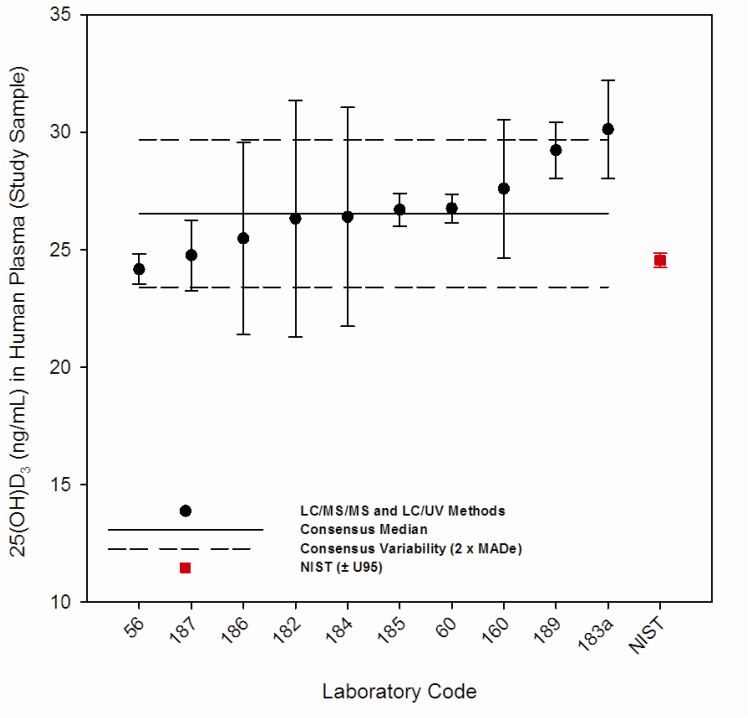


Figure 3. Winter 2010 exercise results for the total 25-hydroxyvitamin D (D_2 + D_3) in the human plasma (study sample)**.**

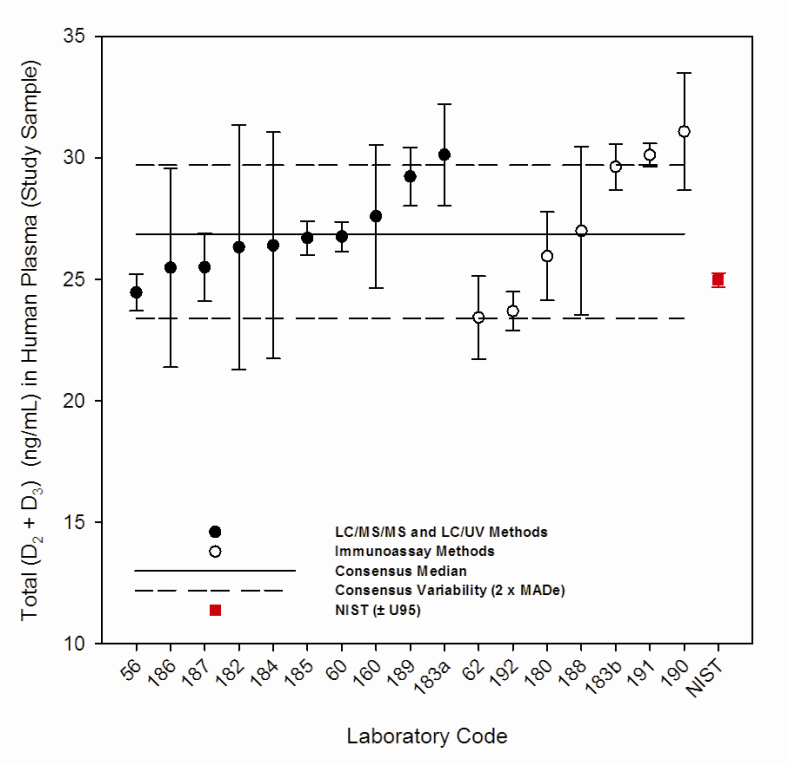
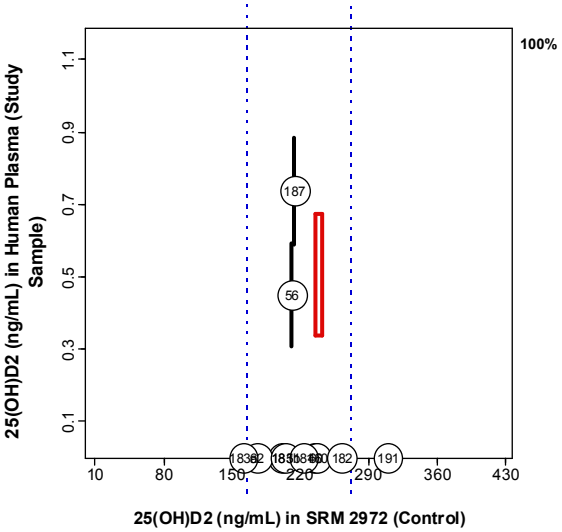


Figure 4. Complete data and summarized results for (a) 25(OH)D_2 and (b) 25(OH)D_3 in both the SRM 2972 (control) and the human plasma (study sample)**.**

(a) 25(OH)D_2

	Control	Study Sample: Human Plasma				
Lab	SRM 2972	A1	B1	C1	Mean	s_{total}
56	211.3	0.5	0.4		0.45	0.07
187	214.	0.74	0.81	0.66	0.74	0.07
185	201.					
60	235.					
62	175.7					
160	237.					
182	262.					
183a	161.2					
183b	205.					
184	223.					
191	309.6					
N	12				2	
Mean	222.95				0.59	
Median	218.50				0.59	
MADe	26.69				1.00	
%RSD	12.2				1.0	
NIST	238.60				0.51	
$\pm U_{95}$	3.90				0.17	



(b) 25(OH)D_3

	Control	Study Sample: Human Plasma				
Lab	SRM 2972	A1	B1	C1	Mean	s_{total}
56	260.4	24.4	23.8	24.3	24.2	0.3
60	324.2	26.5	26.7	27.1	26.8	0.3
160	329.	26.3	29.2	27.3	27.6	1.5
182	368.	24.	26.	29.	26.3	2.5
183a	353.8	31.3	29.8	29.3	30.1	1.0
184	351.	23.8	28.3	27.1	26.4	2.3
185	354.4	26.4	26.7	27.	26.7	0.3
186	333.8	27.6	25.3	23.5	25.5	2.0
187	347.	24.8	24.	25.5	24.8	0.8
189		29.8	29.3	28.6	29.2	0.6
62	340.3					
183b	291.					
191	319.8					
N	12				10	
Mean	331.06				26.76	
Median	337.05				26.55	
MADe	22.76				1.57	
%RSD	6.8				5.9	
NIST	334.80				24.27	
$\pm U_{95}$	5.20				0.75